We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

4,800
Open access books available

122,000
International authors and editors

135M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities

CLARIVATE ANALYTICS BOOK CITATION INDEX INDEXED

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

1

Cryopreservation of Rat Bone Marrow Derived Mesenchymal Stem Cells by Two Conventional and Open-Pulled Straw Vitrification Methods

Mohammad Hadi Bahadori*
Cellular and Molecular Biology Research Center, Faculty of Medical Science, Guilan University of Medical Sciences, Rasht, Iran

1. Introduction

Bone marrow (BM) is a complex tissue containing populations of progenitor and stem cells (1). One type, hematopoietic stem cells (HSCs), can renew circulating blood elements such as red blood cells, monocytes, platelets, granulocytes and lymphocytes. The other is mesenchymal stem cells (MSCs), which possess two important properties of long-term self renewal and differentiate into osteoblasts, chondroblasts, adipocytes and hematopoiesis supporting stroma (2, 3). Their mesenchymal differentiation potential is retained even after repeated subcultivation *in vitro* (4, 5). Besides originating the forming mesenchymal tissue, many studies have demonstrated that MSCs could differentiate into various non-mesenchymal tissue lineages under appropriate experimental conditions *in vitro* and *in vivo*, such as hepatocytes (6, 7), cardiomyocytes (8, 9), lung alveolar epithelium (10), olfactory epithelium (11), inner hair cells (12), neurons and neuroglia (1, 4, 13). MSCs are spindle shaped fibroblast-like cells that are easily isolated, cultured and expanded *in vitro* due to their adherent characteristics, and not associated with any ethical debate (14). Thus, MSCs may be used in the treatment of a diverse variety of clinical conditions (15) such as engraftment of various organs (16). The long-term cultivation of MSCs may fail for many reasons: genotypic drift, senescence, transformation, phenotypic instability, and contamination or incubator failure. The inability to cultivate MSCs will result in the lack of MSCs for both experimental and clinical use (17). Therefore, it is necessary to cryopreserve MSCs as cell seeds. Although increasing telomerase expression of cells may overcome cell senescence (18), cryopreservation of hMSCs may be more practical in order to save time and culture materials (16, 19). Resuscitated MSCs can be subcultivated for many passages without a noticeable loss of viability and capability of osteogenic differentiation (20-22).

Formulating a cryopreservation protocol for hMSCs is required because these cells cannot survive for long periods under *in vitro* culture conditions. Slow rate cooling methods using dimethylsulfoxide (DMSO) as a cryoprotectant have been used for a wide variety of MSC lines established from bone marrow (23, 24), umbilical cord blood (23-25), hematopoietic progenitor cells (26) and mouse ES cell lines (27). In most protocols, cells are suspended in

* Corresponding Author

freezing medium containing DMSO at 5-20%, transferred into glass or plastic cryovials and then frozen by cooling at 1.0 to 2.0 °C/min (28). Slow freezing reduces ice crystal formation and eliminates toxic and osmotic damage to cells through exposure to low concentrations of cryoprotectants while slowly decreasing temperatures (29). However, it is difficult to completely eliminate injury by intracellular ice formation. Damage by ice crystal formation in the cytoplasm during the freezing process is one of the possible causes of cell death; such conventional methods, are not applicable to hMSC cells because many of these cells die immediately after thawing (28). Alternately, vitrification, a rapid cooling method using a high concentration of cryoprotectant, could also be used. Vitrification can completely eliminate damage caused by ice crystal formation in the cytoplasm of cells during freezing (29, 30). It is also advantageous because the procedure takes a relatively short time and a programmable temperature decreasing container is not required (31).

Vitrification has been used for the cryopreservation of oocytes, fertilized eggs and embryos of several mammalian species including humans in order to prevent ice crystal formation (32). There have been some reports demonstrating that embryonic stem (ES) cells could be successfully cryopreserved by vitrification in recent years (27, 33,34). Moon et al. tested vitrification of the human amnion-derived mesenchymal stem cells (HAMs) by using a two-step exposure to equilibration and vitrification solutions (21). They used an EG-based cryoprotectant and their findings were in line with previous reports that showed the superiority of EG. EG has been proven to be less toxic on fibroblast and other somatic cells in comparison with permeating agents such as DMSO and propylene glycol (PROH) that have been used on murine and human embryos (35). However, as a long-term preservation method for HAMs, a well-defined protocol of cryopreservation needs to be established for a human bone marrow derived mesenchymal stem cell bank. In the present study, to confirm the proliferative capability and pluridifferentiation of cryopreserved adult hMSCs; we chose ethylene ficoll sucrose (EFS) 40 that contained 40% v/v EG for the vitrification solution. hMSCs that were cryopreserved for two months were resuscitated and cultivated for 15 passages. An analysis of their expansion, morphological and pluridifferentiation characteristics was undertaken. Finally, under induction conditions, adipogenic and osteogenic potentials have been discussed.

2. Materials and methods

2.1 Preparation and culture of MSCs

For isolation of rat MSCs; female Sprague-Dawley rats (weighing 200-250 g) with the approval from the Institute for Animal Care were obtained from the Animal Center, Faculty of Medicine, Guilan University of Medical Sciences. Rats were killed by intraperitoneal administration of a lethal dose of sodium pentobarbital. The femurs and tibias were carefully dissected away from attached soft tissue as previously reported with modification (1). The ends of the bones were cut, and the bone marrow was aseptically extruded with 5 ml PBS solution by using a syringe with a 21G needle and flushing the shaft ten times. The marrow tissue was dissociated by pipetting. The cell suspension was then centrifuged at 500 × g for 5 minutes and the supernatant was discarded. Bone marrow mesenchymal stem cells (BMSCs) were then mechanically dispersed into a single-cell suspension so that the density of BMSCs reached 10^6 cells/ml. At this point, marrow cells were plated in a 25 cm^2 plastic flask in Dulbecco's modified eagle medium (DMEM) containing 20% fetal bovine serum

(FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. All cells were incubated at 37 °C, in an atmosphere of 5 % humidified CO2. After 48 hours incubation, the nonadherent cell populations were removed and the medium was added and replaced every three or four days for about two weeks. When the cells grew to 80% confluency they were harvested with 0.25% trypsin and 1 mM EDTA (Gibco, UK) for 5 minutes at 37°C, replated and diluted 1:3 on a 25 cm^2 plastic flask, again cultured to the next confluency and harvested. Prior to their use in inducing differentiation and vitrification MSCs that were passaged approximatly 15 times were morphologically evaluated.

2.2 Cryopreservation of MSCs

MSCs at passage 4 of pre-cryopreservation were harvested and centrifuged at 400 × g for 15 minutes as mentioned above. Approximately 1 $\times 10^6$ cells/ml of randomly selected batches were cryopreserved by using the vitrification method or OPS vitrification.

2.3 Vitrification and thawing procedure

MSCs were cryopreserved by using a two-step exposure to the equilibration and vitrification solutions (34). The equilibration solution was 20% ethylene glycol (EG; Sigma) and the vitrification solution was composed of 40% EG, 18% Ficoll - 70 (Sigma) and 0.3 M sucrose (Sigma). All solutions were based on PBS (Sigma) containing 20% FBS. A pellet of ~1 × 10^6 MSCs (~10 µl) was first suspended in 50 µl equilibration solution for 5 minutes and then mixed with 500 µl vitrification solution for 40 seconds. Suspended MSCs were immediately transferred to 1.2 ml cryovials (Nunc) and plunged directly into liquid nitrogen. The OPS vitrification method was carried out according to Reubinoff et al. (33). For OPC, a pellet of ~1 × 10^6 MSCs (~10 µl) was first suspended in 50 µl equilibration solution for 5 minutes and then mixed with 500 µl vitrification solution for 40 seconds. Suspended MSCs were at once transferred to 0.25 ml plastic straws (IMV, L'Aigle, France). Immediately afterwards, the straws were immersed in liquid nitrogen for two months. Following storage, the cells were thawed by rapidly immersing the vials and straws in a water bath at 37 °C. After warming for about 7 seconds, (at approx. 1800 °C/min) the contents of the vials and straws were suspended serially in 0.5, 0.25 and 0 M sucrose in PBS containing 20% FBS. After thawing, the survival rate was evaluated by the trypan blue staining method. After removing some of the cell pellet and adding 0.4% trypan blue (Sigma), the cells were plated onto a slide and unstained cells were counted as live cells (26). The remaining cells were centrifuged at 200 × g for 10 minutes and washed three times with DMEM medium supplemented with 20% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were immediately plated at a density of 1 × 10^6 cells/ml in a 25 cm^2 culture flask and subcultured over seven days in the above described condition.

2.4 Evaluation of the differentiation potential of cryopreserved MSCs

2.4.1 Adipogenic induction

Pre and post-cryopreserved MSCs were seeded on coverslips in a six-well plate and cultured in DMEM with 10% FBS. Cells with nearly 80% confluency were exposed to DMEM supplemented with 5µg/ml insulin, 1 µM dexamethasone, 100 nM indomethacine, 0.5 mM methylisobutylxanthine (Sigma), and 10% FBS for 48 hours. Cells were then incubated in the same medium without dexamethasone. For control, cells were cultured in regular medium

as above. The medium was changed every third or fourth day. One week after induction, adipogenic differentiation was evaluated by the cellular accumulation of neutral lipid vacuoles that were stained with oil-red O (Sigma) and observed under an inverted microscope (17). Briefly, after fixation in 5% metanol, induced MSCs were stained in filtered oil red O for 2-3 hours and then rinsed with 60% isopropyl alcohol.

2.4.2 Osteogenic induction

To identify osteogenic differentiation, thawed and non-cryopreserved MSCs were cultured in 100 nM dexamethasone, 10 mM ß-glycerol phosphate and 50 µM ascorbic acid-2-phosphate in 400 µl DMEM-LG supplemented with 10% FBS on coverslips in a six-well plate for subsequent staining. During the culture period, the medium was changed once per week. After 14 days, osteogenic differentiation was evaluated by staining the coverslips with fresh 0.5% alizarin red solution (1).

2.4.3 Colony-forming unit assays

For these assays, both thawed and non-cryopreserved cells were plated at 1×10^6 cells per ml and cultured for 14 days in 25 cm^2 tissue culture flasks. After 14 days, the cultures were stained with giemsa for 5 minutes. The formations of colonies were considered acceptable until passage 15 (P15) and those less than 2 mm in diameter or faintly stained were excluded.

2.4.4 Statistical analysis

Statistical analysis for comparison of the postthaw survival rate was performed using the χ^2 test. Statistically significant values were defined as $p<0.05$. All experiments were conducted in triplicate.

3. Results

The growth and morphology of MSCs appeared rather heterogeneous in primary culture as seen in Fig 1A. Under a phase contrast microscope, the cells appeared fibroblast-like, elongated and spindle shaped with a single nucleus (Fig 1B). These cells showed the ability to form colonies with the occasional cell sphere formation giving an impression of embryoid bodies (Fig 1C). However, they progressively showed homogenous fibroblast-like features following subsequent subculture (Fig 1D).

3.1 Morphology and growth of vitrified-thawed MSCs

The duration of storage in frozen state for MSCs was two months. Post-cryopreserved MSCs from both the vitrification method and OPS vitrification had similar cellular morphology and colony-formation. Resuscitated MSCs first grew as isolated colonies after initial plating. Subsequently these adherent cells grew as typically fibroblastic or spindle shaped.

As the cells approached confluence, they assumed a more spindle-shaped, fibroblastic morphology (Fig 2A, B). The thawed and non-cryopreserved MSCs were subcultured until P15. Until the P9, fibroblast-like morphology was consistently observed in both the thawed and non-cryopreserved MSCs. At the P10, cells in both cultures became large and flat, suggesting senescence.

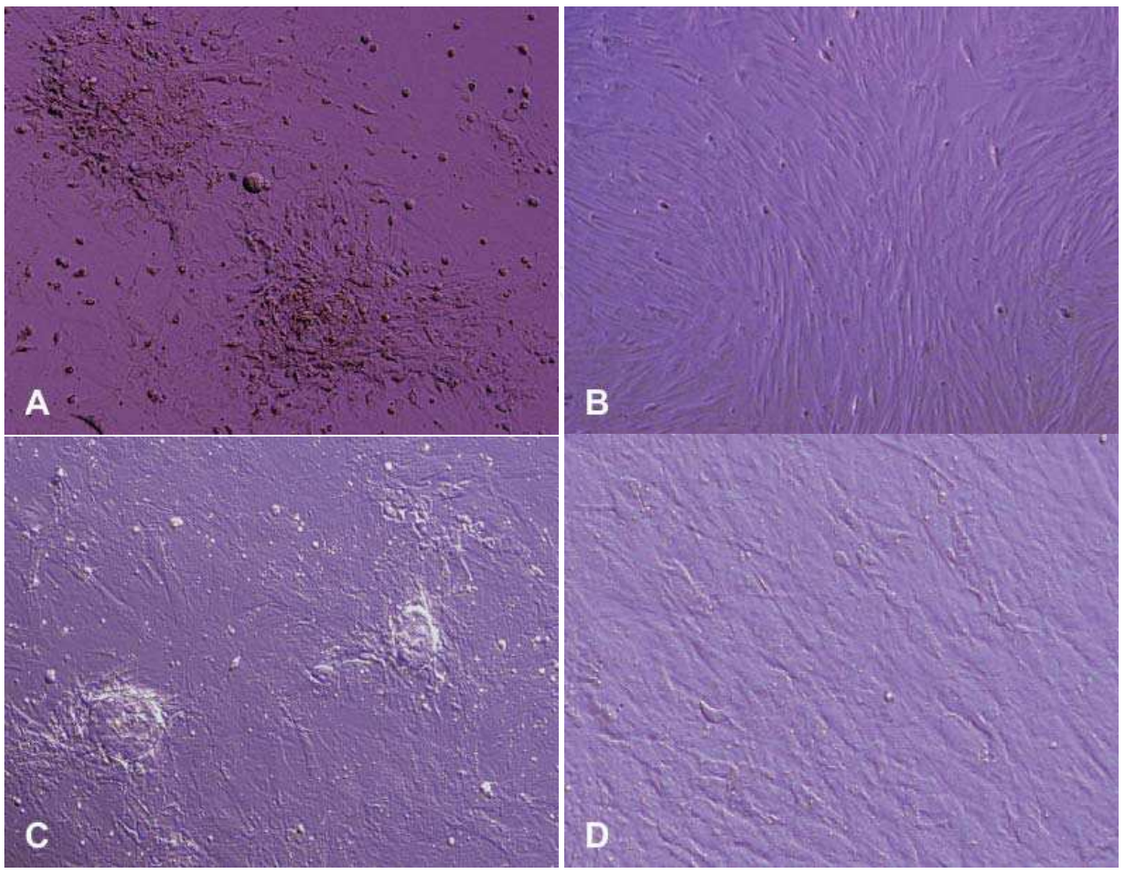


Fig. 1. Morphology and growth of MSCs. (A) Primary (x 40), (B) Passage 4 (14 days, x 100), (C) Passage 4 (35 days, x 100) and (D) Passage 4 (40 days, x 100). In primary culture, cell growth was scattered with some colony formation. Followin subsequent subculture, the cells changed into spindle-like fibroblasts.

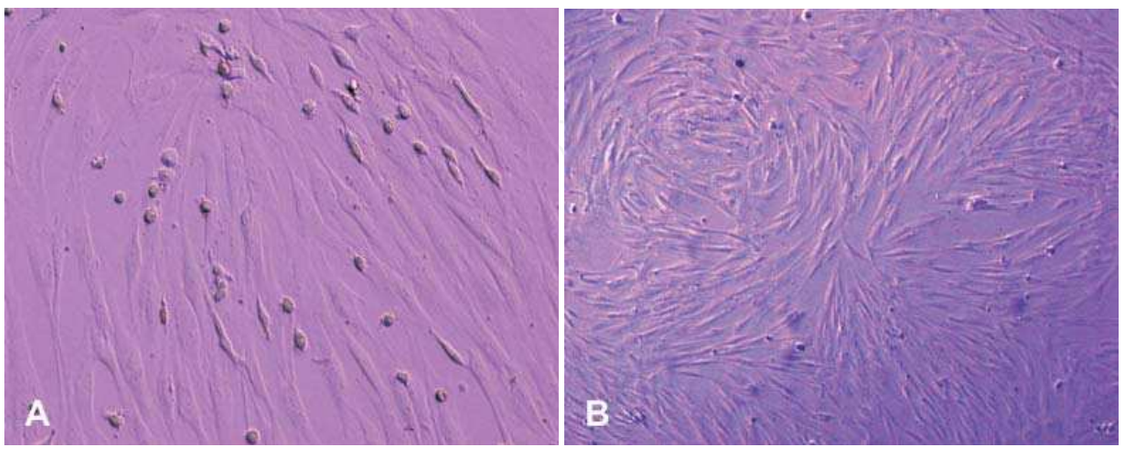


Fig. 2. Morphology and growth of vitrified-thawed MSCs. Phase contrast images of MSCs two months after thawing from: (A) vitrification method (x 100) and (B) OPS vitrification (x 40). MSCs had a similar morphology to fibroblasts and were indistinguishable from non-cryopreserved MSCs.

4. Viability of vitrified-thawed MSCs

Live/dead viability of MSCs was determined by the trypan blue staining test. The number of MSCs was counted and compared to that of the control. After thawing, the viability rates were 81.33% ± 6.83 for the vitrification method and 80.83% ± 6.4 for the OPS vitrification, while values with the pre-vitrification control group were 88.16% ± 6.3, respectively (Table 1). There were no differences in viability between them.

No.	Before vitrification (%)	Vitrification method (%)	OPS method (%)
1	85	81	82
2	93	92	90
3	78	77	70
4	96	81	79
5	89	72	81
6	88	85	83
Mean ± SD	88.16(SD ± 6.30)	81.33 (SD ± 6.83)	80.83(SD ± 6.40)

OPS: open-pulled straw, student t-test ($p<0.05$).

Table 1. Percentage of cell viability of non-cryopreserved and two different cryopreserved vitrification methods by the trypan blue staining test.

5. Colony forming unit assay

For these assays, cells of both thawed and noncryopreserved MSCs or cultures were plated at 1×10^6 cells/ml and MSCs in culture showed a colony formation consisting of 40-80 cells in 25 cm^2 flasks for 14 days (Fig 3). After P9, the cells showed no colony forming ability which illustrated that colony forming ability decreases with increasing passages.

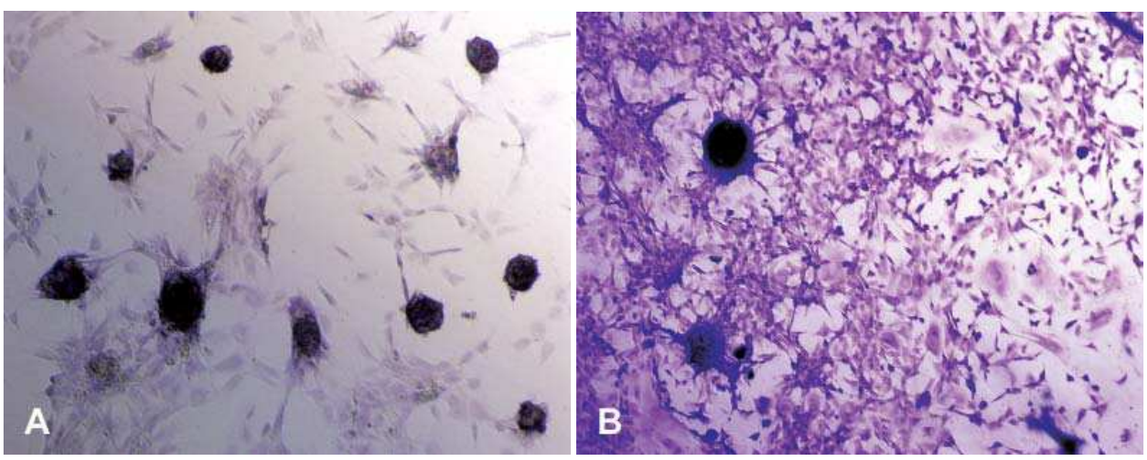


Fig 3. Phase contrast images of typical MSCs colony morphology before (A) and after (B) cryopreservation. Cell sphere formation in the MSCs culture produced colony formation units that stained with giemsa (A and B: x 40).

6. Differentiation of post-cryopreserved MSCs

After culturing for adipogenic differentiation, the accumulations of numerous neutral lipid vacuoles were detectable in the cytoplasm of vitrified-thawed cells (Fig 4A). Following three weeks of induction, oil Red O staining showed the lipid droplets with orange red color, which demonstrated the committed differentiation of MSCs into adipocytes (Fig 4B). The control cells showed no detectable lipid vacuoles. Under culture with osteogenic induction medium, resuscitated MSCs detached and floated in the medium. After three weeks, mineral accumulations were observed by alizarin red staining (Fig 5A, B). Similar results were observed in the group of non- cryopreserved MSCs when osteogenically induced under the same condition (Fig 5C, D).

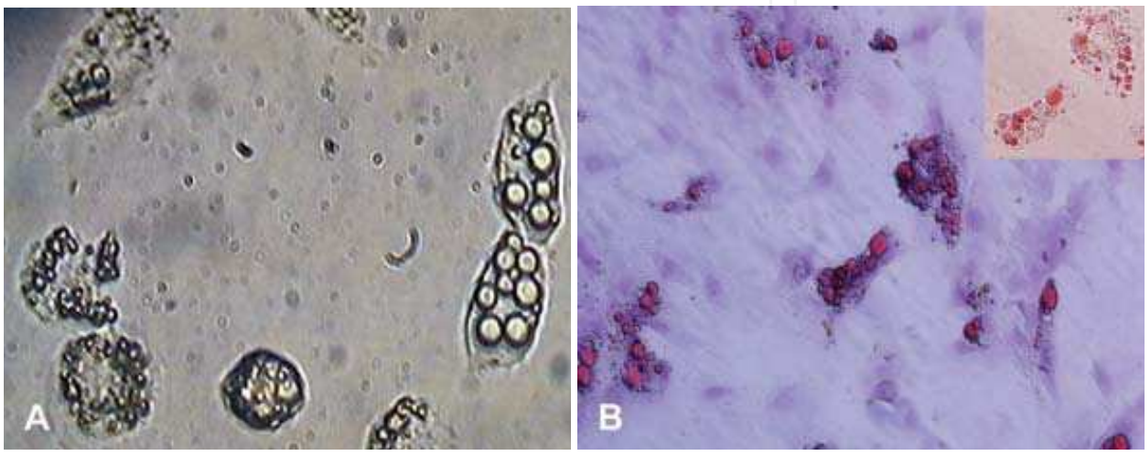


Fig 4. Evaluation of adipogenesis potential of MSCs under a phase-contrast microscope. Both the non cryopreserved MSCs and vitrified-thawed MSCs after treatment by adipogenic medium showed numerous neutral lipid vacuoles which accumulated in the cytoplasm. (A) Confirmed by oil red O staining (oil red O + hematoxiline, which one is the top right one: oil red without hematoxiline, x 100), (B).

7. Discussion

Cryopreservation is an important method to maintain cells for biological research and medical applications such as tissue engineering, gene therapy, cell transplantation, pharmacological testing and future therapeutic indications (17, 28). A study on the long-term storage of BM-derived MSCs is of critical importance (1). The objective of the current investigation was to test the possibility that vitrification could be a useful method for the cryopreservation of MSCs. Thus, in the present study, we isolated MSCs from bone marrow of adult female rats. In culture; MSCs are characterized by their capacity to adhere to a plastic culture surface and form a fibroblast-like shape (Fig 1). Our data corroborated previous findings from other groups which showed homogenous fusiform features with oval vesicular nuclei (36) and the colony forming ability of MSCs, which decreased with increasing passages (37). The achievement of pure fibroblastic clones from murine bone marrow was first reported by Wang and Wolf (38).

Furthermore, Eslaminejad et al. obtained an average of 15-17 clones, each one consisting of several fibroblastic cells per 24-well plate (39). This approach yielded both the number and

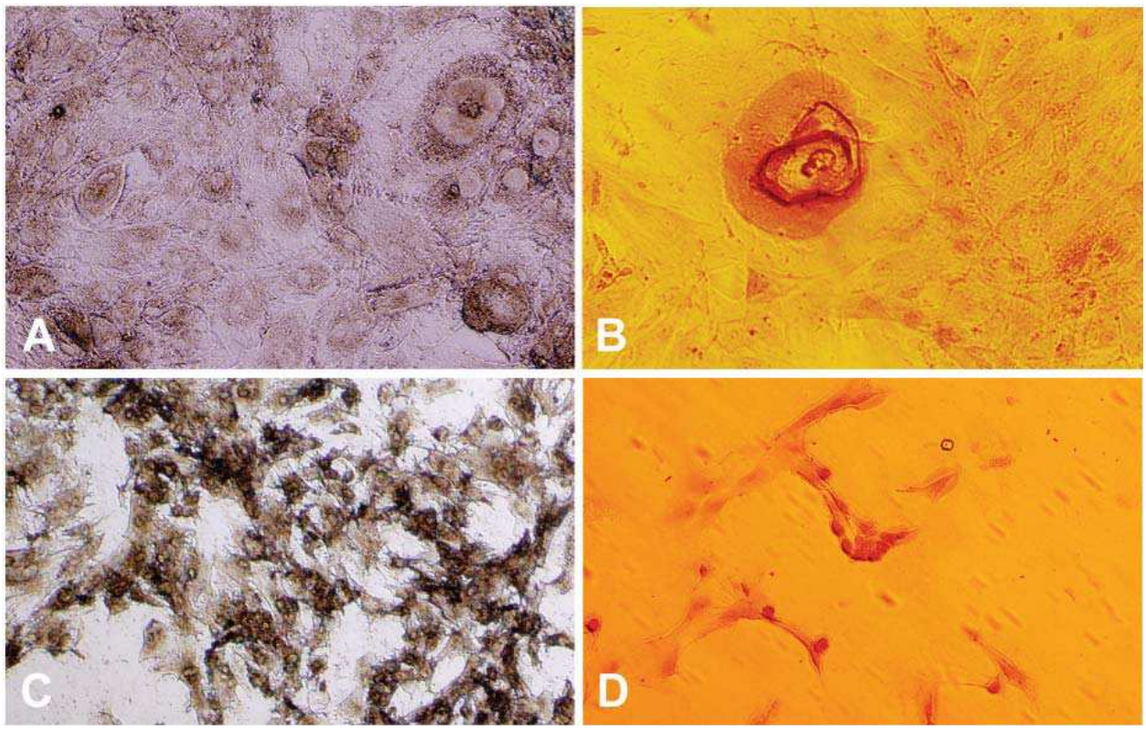


Fig 5. Differentiation potency of nonvitrified and vitrified-thawed MSCs observed under a phase-contrast microscope. (A) Nonvitrified MSCs after osteogenic induction. (B) Alizarin red staining, x 100. (C) Vitrified MSCs after osteogenic induction. (D): Alizarin red staining, x 100. After induction of differentiation for 2-3 weeks in respective induction media, the mineralized extracellular matrix of pre and post-vitrification MSCs stained positively with alizarin red (arrows).

cellular densities of colonies that were dependent upon the number of the cells plated per culture dish and was the method employed in this investigation. We also successfully vitrified MSCs and confirmed the morphology, viability rate and differentiation capacity of post-vitrification MSCs as compared with non-cryopreserved controls. Generally, most comprehensive studies on the cryopreservation of MSCs were carried out by using slow-rate cooling methods (17, 22-24). Slow-rate cooling methods using dimethylsulfoxide (DMSO) as a cryoprotectant are effective for a wide variety of cell lines, including ES cell lines (27, 28). This method by decreasing temperature slowly with a low concentration of cryoprotectant is used to balance damage caused by various factors including ice crystal formation, fracture, toxic and osmotic damage (29).

It is difficult to completely eliminate injuries from intracellular ice formation, which is the main source of fracture and damage to the cytoplasm (29). It is also a time-consuming procedure and requires an expensive programmable freezer (31). On the other hand, there are some reports that stem cells are highly sensitive to cryoinjury and the vitrification method is a better choice for HES cell cryopreservation than conventional slow freezing and rapid thawing (28, 33). This method has been previously applied for cryopreservation of oocytes, fertilized eggs and embryos of several mammalian species, including humans (32).

In vitrification method, the concentrations of cryoprotectants seem to be dangerously high at the final phases, it happens at low temperatures, where the real toxic effect is minimal. Moreover, the high cooling and warming rates applied at vitrification provide an unique benefit compared to the traditional freezing (40).

Umbilical cord blood-derived mesenchymal stem cells (UCB-derived MSCs) were vitrified by vitrification and by programmed freezing without dimethyl sulfoxid (DMSO) by Wang et al. (2011). Their results showed that the viability of thawed UCB-derived MSCs was enhanced from 71.2% to 95.4% in the presence polyvinyl alcohol (PVA) for vitrification, but only < 10% to 45% of viability was found for programmed freezing (41). While, Kim et al. reported that Post-thaw colony-formation of embryonic stem cells (ESCs) was detected only after a slow freezing using DMSO by stepwise placement of a freezing container into a -80°C deep freezer and subsequently into -196°C liquid nitrogen, while no proliferation was detected after vitrification (42). Also, hMSCs from pre- and post-cryopreservation by slow freezing had similar colony-formation and cellular morphology similar to our resuls (17). Low survival of human ESCs has been also reported when they are frozen slowly with DMSO (43). There have been several reports to demonstrate the superiority of vitrification to other freezing programs for human ESCs, because it is able to avoid cell injury resulted from ice crystal formation (42, 44).Carvalho et al. (2008) reported that viability of frozen BM-MSCs by slow freezing which was to added 5% DMSO was 94.76% and 90.58% viability before versus after cryopreservation (45). Also, the high survival rate (81.8%) is obtained after cryopreservation of human ESCs by programmed freezing (46). Our result showed 81.33% and 80.83% cell viability of two different cryopreserved vitrification methods versus 88.16% viability of non-cryopreserved cells. Nevertheless, we are not able to deny the feasibility of vitrification program for effective cryopreservation of SCs (42).

Also Fujioka et al. vitrified ESCs using EFS40; ethylene glycol, 18% ficoll 70,000 MW and 0.3 M sucrose (similar to this work) and slow-freezed in freezing medium containing 10% DMSO. Their results indicated that the vitrification methods yielded higher cell recovery and survival rates than did the slow-rate freezing methods (28).

DMSO has been well known as a toxic agent for stem and progenitor cells, and particularly for human embryonic stem cells. It is also known as a powerful differentiation agent that may interact with the chromatin structure (47).

Therefore, the selection of suitable cryoprotectants is essential. Cryopreservation procedure ought to become different according to cell type and cellular characteristics. So, all components consisting of freezing and thawing procedures should specifically be determined in each case of cryopreservation (42).

This study was DMSO-free vitrification of MSCs using cryovial and straw. Straws, preferably thin straws, even in sealed form, can be cooled safely with an increased cooling rate (40). There was any report about vitrification of MSCs without DMSO.

So that in the present experiment, we have observed the viability and proliferation capability as well as differentiation potential of cryopreserved MSCs *in vitro*. Inverted microscope findings showed that, after culturing for seven days, numerous MSCs adhered well to the surface culture dish (Fig 1). In a study by Moon et al. on human amnion-derived mesenchymal stem cells (HAMs), they observed that slow freezing resulted in a lower

survival rate compared with vitrification, indicating a high efficiency of the vitrification procedure (21).

Moreover, Heng (48) demonstrated that 39.8 ± 0.9% of the hMSCs could be recovered after cryopreservation using a conventional slow freezing method which was lower than that our result (Table 1). Here, resuscitated MSCs kept a high proliferative potential. They first grew as clones after limiting dilution and then expanded rapidly with the typical features of spindle-shaped cell bodies and confluence after a lag phase of 6-14 days. There were no differences among the pre and post-cryopreservation of colony formation at the same passage (Fig 2). In addition, the results showed that the passage procedure was selective for MSCs and it could be inferred that the passaging of resuscitated MSCs increased cellular homogeneity.

Ji et al. demonstrated that cryopreservation of encapsulated HES cells offers better cellular viability, higher colony recovery, and less differentiation than the slow-freezing techniques most commonly used to preserve HES colonies. Therefore, this difference in recovery may be due to differences in cell lines, freezing and thawing protocols, or growth substrate (49).

On the other hand, previous studies have shown that cyropreservation had no effect on either the proliferation or osteogenic and adipogenic differentiation of human MSCs *in vitro* (5, 22). In agreement with these reports, Liu et al. using slow cooling with Me2SO as a cryoprotectant and rapid thawing demonstrated that thawed cryopreserved human MSCs had higher survival rates in comparison with non-cryopreserved MSCs and differentiated into osteoblasts when cultured in osteogenic media. Also they found that cryopreserved hMSCs could not differentiate into osteoblasts spontaneously when cultured in basic culture media (50). In addition to the characteristics described above, our present study demonstrated that post-cryopreserved MSCs from bone marrow were still pluripotential and differentiated into osteoblasts and adipocyte under appropriate culture conditions (Fig 3, 4). These observations suggest that the "memory" of proliferation and differentiation in MSCs is not affected by the process of vitrification. The ability of frozen BM-MSCs by slow freezing to differentiate into mesenchymal derivatives (such as osteogenic and adipogenic) reported by Lee et al. (4). In this study, we established a two-step vitrification protocol for MSCs using EFS containing 40% v/v EG for the vitrification solution, which is widely used for successful vitrification of mouse embryos (30), human blastocysts (32) and ESCs (34). Our findings are in line with the reports by Gajda et al. who used the same methods for somatic cells which have been proven to be less toxic on bovine skin fibroblast and cumulus cells (35).

EG is the most commonly used cryoprotectant for vitrification due to its low molecular weight and low toxicity (35). In addition, additives with high molecular weights, such as sucrose, can significantly reduce toxicity by decreasing the concentration of permeating agents required for the vitrification solution. We also used ficoll as a macromolecule to promote permeation by cryoprotectants, which seems to have the advantages of lower toxicity, higher solubility and lower viscosity (30). In a study by Moon et al. on HAMs, they observed that the combination of EG with either PROH or DMSO resulted in a very low survival rate of HAMs as compared with EFS alone (21). Also, Kuleshova and Lopata ascertained the advantages of vitrification when compared with earlier applied cryopreservation techniques (51). These advantages include the control of solute penetration

and dehydration rates, prevention of prolonged temperature shock and damage from ice formation, and inexpensive equipment and running costs. Vitrification is a process where glass-like solidification of a solution occurs without the formation of ice crystals inside living cells, by exposure to a high concentration of cryoprotectant with a higher cooling rate (52). This procedure is a simple method to circumvent the obstacles of slow freezing without the need for a freezing container to modulate the reduction of temperature in a deep freezer before storing in liquid nitrogen (21).

8. Conclusion

In the present experiment, it was shown that vitrification can be an efficient storage method for MSCs without losing their activity and usual properties. Such a system will be exceedingly helpful for both experimental research and medical applications.

9. Acknowledgments

This research was a part of proposal supported by a grant (3/132/8162 /P) funded by the Guilan University of Medical Sciences and was done in the Cellular and Molecular Research Center at the Faculty of Medicine. The authors wish to thank both centers. There is no conflict of interest in this article.

10. References

[1] Eslaminejad MB, Nazarian H, Taghiyar L. Mesenchymal stem cells isolation from removed medium of rat's bone marrow primary culture and their differentiation into skeletal cell lineage. Yakhteh. 2008; 10(1): 62-72.

[2] Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells. 2001; 19(3): 180-192.

[3] Wu QY, Li J, Feng ZT, Wang TH. Bone marrow stromal cells of transgenic mice can improve the cognitive ability of an Alzheimer's disease rat model. Neurosci Lett. 2007; 417(3): 281-285.

[4] Lee MW, Choi J, Yang MS, Moon YJ, Park JS, Kim HC, et al. Mesenchymal stem cells from cryopreserved human umbilical cord blood. Biochem Biophys Res Commun. 2004; 320(1): 273-278.

[5] Bruder SP, Jaiswal N, Haynesworth SE, Growth kinetics, self renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. J Cell Biochem. 1997; 64(2): 278-294.

[6] Kang XQ, Zang WJ, Song TS. Xu XL, Yu XJ, Li DL, et al. Rat Bone marrow mesenchymal stem cells differentiation into hepatocytes *in vitro*. World J Gastroenterol. 2005; 11 (22): 3479-3484.

[7] Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz- Gonzalez XR, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature. 2002; 418: 41-49.

[8] Baharvand H, Mathae KI. Culture condition difference for stablishment of new embryonic stem cell lines from the C57 BL/6 and BALB/c mouse strains. *In Vitro* Cell Dev Biol Anim. 2004; 40(3-4): 76-81.

[9] Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation. 2002; 105: 93-98.

[10] Kotton DN, Ma BY, Cardoso WV, Sanderson EA, Summer RS, Williams MC, et al. Bone marrow-derived cells as progenitors of lung alveolar epithelium. Development. 2001; 128: 5181-5188.

[11] Tsujigiwa H, Nishizaki K, Teshima T, Takeda Y, Yoshinobu J, Takeuchi A, et al. The engraftment of transplanted bone marrow-derived cells into the olfactory epithelium. Brain Res. 2005; 1052(1): 10-15.

[12] Jeon SJ, Oshima K, Heller S, Edge AS. Bone marrow mesenchymal stem cells are progenitors *in vitro* for inner ear hair cells. Mol Cell Neurosci. 2007; 34(1): 59-68.

[13] Suzuki H, Taguchi T, Tanaka H, Kataoka H, Li Z, Muramatsu K, et al. Neurospheres induced from bone marrow stromal cells are multipotent for differentiation into neuron, astrocyte, and oligodendrocyte phenotypes. Biochem Biophys Res Commun. 2004; 322(3): 918-922.

[14] Alviano F, Fossati V, Marchionni C, Arpinati M, Bonsi L, Franchina M, et al. Term Amniotic membrane is a high throughout source for multipotent mesenchymal stem cells with the ability to differentiate into endothelial cells *in vitro*. BMC Dev Biol. 2007; 7:11.

[15] Korbling M, Estrov Z. Adult stem cells for tissue repair. A new therapeutic concept? N Engl J Med. 2003; 349(6): 570-582.

[16] Liechty KW, MacKenzie TC, Shaaban AF, Radu A, Moseley AM, Deans R, et al. Human mesenchymal stem cells engraft and demonstrate site-specific differentiation after in utero transplantation in sheep. Nat Med. 2000; 6(11): 1282-1286.

[17] Xiang Y, Zheng Q, Jia BB, Huang GP, Xu YL, Wang JF, et al. Ex vivo expansion, adipogenesis and neurogenesis of cryopreserved human bone marrow mesenchymal stem cells. Cell Biol Int. 2007; 31(5): 444-450.

[18] Poh M, Boyer M, Solan A, Dahl SL, Pedrotty D, Banik SS, et al. Blood vessels engineered from human cells. Lancet. 2005; 365(9477): 2122-2124.

[19] Guy JA, Wouters R, Successful vitrification of umbilical cord tissue to harvest stem cell for therapies in the future. J Cryobiol/abstract. 2008; 57: 329.

[20] Perry BC, Zhou D, Wu X, Yang FC, Byers MA, Chu TM, et al. Collection, cryopreservation, and characterization of human dental pulp derived mesenchymal stem cells for banking and clinical use. Tissue Eng Part C Methods. 2008; 14(2): 149-156.

[21] Moon JH, Lee JR, Jee BC, Suh CS, Kim SH, Lim HJ, et al. Successful vitrification of human amnionderived mesenchymal stem cells. Hum Reprod 2008; 23(8): 1760-1770.

[22] Kotobuki N, Hirose M, Machida H, Katou Y, Muraki K, Takakura Y, et al. Viability and osteogenic potential of cryopreserved human bone marrow derived mesenchymal cells. Tissue Eng. 2005; 11(5-6): 663-673.

[23] Haack- Sorensen M, Bindslev L, Mortensen S, Friis T, Kastrup J. The influence of freezing and storage on the characteristics and functions of human mesenchymal stromal cells isolated for clinical use. Cytotherapy 2007; 9(4): 328-337.

[24] Lee RH, Kim B, Choi I, Kim H, Choi HS, Suh K, et al. Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. Cell Physiol Biochem. 2004; 14: 311- 324.

[25] Romanov YA, Svintsitskaya VA, Smirnov VN. Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. Stem Cells. 2003; 21(1): 105-110.

[26] Zhao J, Hao HN, Thomas RL, Lyman WD. An efficient method for the cryopreservation of fetal human liver hematopoeitic progenitor cells. Stem Cells. 2001; 19(3): 212-218.

[27] Yang PF, Hua TC, Wu J, Chang ZH, Tsung HC, Cao YL. Cryopreservation of human embryonic stem cells: a protocol by programmed cooling. Cryo Letters. 2006; 27(6): 361-368.

[28] Fujioka T, Yasuchika K, Nakamura Y, Nakasuji N, Suemori H. A simple and efficient cryopreservation method for primate embryonic stem cells. Int J Dev Biol. 2004; 48(10): 1149-1154.

[29] Vajta G, Nagy Z. Are programmable freezers still needed in the embryo laboratory? Review on vitrification. Reprod Biomed Online. 2006; 12(6): 779-796.

[30] Kasai M, Mukaida T. Cryopreservation of animal and human embryos by vitrification. Reprod Biomed Online. 2004; 9(2): 164-170.

[31] Karlsson JO. Cryopreservation: freezing and vitrification. Science. 2002; 296(5568): 655-656.

[32] Cho HJ, Son WY, Yoon SH, Lee SW, Lim JH. An improved protocol for dilution of cryoprotectants from vitrified human blastocysts. Hum Reprod. 2002;17(9): 2419-2422.

[33] Reubinoff BE, Pera MF, Vajta G, Trounson AO. Effective cryopreservation of human embryonic stem cells by the open pulled straw vitrification method. Hum Reprod. 2001; 16(10): 2187-2194.

[34] Nie Y, Bergendahl V, Hei DJ, Jones JM, Palecek SP. Scalable culture and cryopreservation of human embryonic stem cells on microcarriers. Biotechnol Prog. 2009; 25(1): 20-31.

[35] Gajda B, Katska- Ksiazkiewicz L, Rynska B, Bochenek M, Smorag Z. Survival of bovine fibroblasts and cumulus cells after vitrification. Cryo letters. 2007; 28(4): 271-279.

[36] Stock P, Staege MS, Muller LP, Sgodda M, Volker A, Volkmer I, et al. Hepatocyte Derived from adult stem cells. Transplant Proc. 2008; 40(2): 620-623.

[37] Polisetty N, Fatima A, Madhira SL, Sangwan VS, Vemuganti GK. Mesenchymal cells from limbal stroma of human eye. Mol Vis. 2008; 14: 431-442.

[38] Wang QR, Wolf NS. Dissecting the hematopoietic microenvironment. Clonal isolation and identification of cell types in murine CFU-F colonies by limiting dilution. Exp Hematol. 1990; 18(4): 355-359.

[39] Eslaminejad MB, Nikmahzar A, Taghiyar L, Nadri S, Massumi M. Murine mesenchymal stem cells isolated by low density primary culture system. Dev Growth differ. 2006; 48(6): 361-370.

[40] Vajta G, Kuwayama M. Improving cryopreservation systems. Theriogenology. 2006; 65: 236–244.

[41] Wang HY, Lun ZR, Lu SS. Cryopreservation of umbilical cord blood-derived mesenchymal stem cells without dimethyl sulfoxide. Cryo Letters. 2011; 32(1): 81-88.

[42] Kim GA, Lee ST, Lee EJ, Choi JK, Lim JM. Simplified slow freezing program established for effective banking of embryonic stem cells. Asian-Aust J Anim Sci. 2009; 22(3): 343-349.

[43] Katkov MS, Kim R, Bajpai YS, Altman M, Mercola JF, Loring AV, et al. Cryopreservation by slow cooling with DMSO diminished production of Oct-4 pluripotency marker in human embryonic stem cells. Cryobiol. 2006; 53:194-205.

[44] Suemori H, Yasuchika K, Hasegawa K, Fujioka T, Tsuneyoshi N, Nakatsuji N. Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. Biochem Biophys Res Commun. 2006; 345:926-932.

[45] Carvalho KAT, Cury CC, Oliveira L, Cattaned RII, Malvezzi M, Francisco JC, et al. Evaluation of Bone Marrow Mesenchymal Stem Cell Standard Cryopreservation Procedure Efficiency. Transplant Proc. 2008; 40: 839–841.

[46] Yang PF, Hua TC, Tsung HC, Cheng QK, Cao YL. Effective cryopreservation of human embryonic stem cells by programmed freezing. Conf Proc IEEE Eng Med Biol Soc. 2005; 1: 482-485.

[47] Katkov II, Kan NG, Cimadamore F, Nelson B, Snyder EY, Terskikh AV. DMSO-Free Programmed Cryopreservation of Fully Dissociated and Adherent Human Induced Pluripotent Stem Cells. Stem Cells Int. 2011;2011:981606.

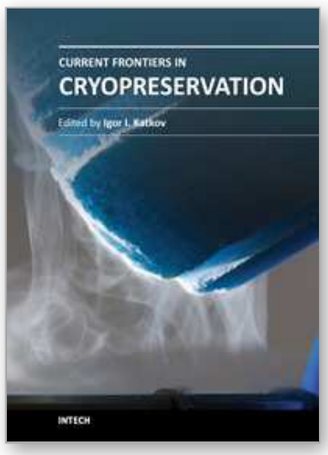
[48] Heng BC. Effect of Rho-associated kinase (ROCK) inhibitor Y-27632 on the post-thaw viability of cryopreserved human bone marrow- derived mesenchymal stem cells. Tissue Cell. 2009;41(5):376-380.

[49] Ji L, de Pablo JJ, Palecek SP. Cryopreservation of adherent human embryonic stem cells. Biotechnol Bioeng. 2004; 88(3): 299-312.

[50] Liu G, Shu C, Cui L, Liu W, Cao Y. Tissue-engineered bone formation with cryopreserved human bone marrow mesenchymal stem cells. Cryobiology. 2008; 56(3): 209-215.

[51] Kuleshova L, Lopata A. Vitrification can be more favorable them slow cooling. Fertil Steril. 2002; 78: 449-454.

[52] Chian RC, Kuwayama M, Tan L, Tan J, Kato O, Nagai T. High survival rate of bovine oocytes matured *in vitro* following vitrification. J Reprod Dev. 2004; 50: 685-696.



Current Frontiers in Cryopreservation

Edited by Prof. Igor Katkov

ISBN 978-953-51-0302-8
Hard cover, 462 pages
Publisher InTech
Published online 14, March, 2012
Published in print edition March, 2012

Almost a decade has passed since the last textbook on the science of cryobiology, Life in the Frozen State, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Mohammad Hadi Bahadori (2012). Cryopreservation of Rat Bone Marrow Derived Mesenchymal Stem Cells by Two Conventional and Open-Pulled Straw Vitrification Methods, Current Frontiers in Cryopreservation, Prof. Igor Katkov (Ed.), ISBN: 978-953-51-0302-8, InTech, Available from: http://www.intechopen.com/books/current-frontiers-in-cryopreservation/cryopreservation-of-rat-bone-marrow-derived-mesenchymal-stem-cells-by-two-conventional-and-open-pull

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen